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APPLICATION NUMBER: ***60/426,111***
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Practitioner's Docket No. 58392-P (70305) ***PATENT***

Preliminary Classification:
Proposed Class:
Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. Section 601, 7th ed.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Jeffrey Keller TEUMER, Jizeng QIAO, Erica LaFONT, and Richard Gregory WOLOWACZ

For: HAIR INDUCTIVE CELL CULTIVATION

BOX PROVISIONAL APPLICATION
Commissioner for Patents
Washington, D.C. 20231

COVER SHEET FOR FILING PROVISIONAL APPLICATION

CERTIFICATION UNDER 37 C.F.R. SECTION 1.10*
(Express Mail label number is mandatory.)
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I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on November 14, 2002, an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. Section 1.10, Mailing Label Number EV097093422US **BOX PROVISIONAL APPLICATION,** Commissioner for Patents, Washington, D.C. 20231.

Donna R. Davis
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(37 C.F.R. SECTION 1.51(c)(1))

WARNING: *"A provisional application must also include the cover sheet required by Section 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this Section." 37 C.F.R. Section 1.53(c)(1). See also M.P.E.P. Section 201.04(b), 6th ed,. rev. 3.*

NOTE: *"A complete provisional application does not require claims since no examination on the merits will be given to a provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application." Notice of December 5, 1994, 59 Fed. Reg. 63,951, at 63,953.*

"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

NOTE: *"A provisional application is not entitled to the right of priority under 35 U.S.C. Section 119 or 365(a) or Section 1.55, or to the benefit of an earlier filing date under 35 U.S.C. Section 120, 121 or 365(c) or Section 1.78 of any other application. No claim for priority under Section 1.78(a)(3) may be made in a design application based on a provisional application. No request under Section 1.293 for a statutory invention registration may be filed in a provisional application. The requirements of Sections 1.821 through 1.825 regarding application disclosures containing nucleotide and/or amino acid sequences are not mandatory for provisional applications." 37 C.F.R. Section 1.53(c)(3).*

NOTE: *"No information disclosure statement may be filed in a provisional application." 37 C.F.R. Section 1.51(d). "Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 Fed. Reg. 63,591, at 63,594.*

NOTE: *"No amendment other than to make the provisional application comply with the patent statute and all applicable regulations, may be made to the provisional application after the filing date of the provisional application. " 37 C.F.R. Section 1.53(c).*

NOTE: *35 U.S.C. 119(e) provides that "[i]f the day that is 12 months after the filing date of a provisional application falls on a Saturday, Sunday, or Federal Holiday within the District of Columbia, the period of pendency of the provisional application shall be extended to the next succeeding secular or business day."*

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. Section 1.51(c)(1)(i). The following comprises the information required by 37 C.F.R. Section 1.51(c)(1):

1. The name(s) of the inventor(s) is/are (37 C.F.R. Section 1.51(c)(1)(ii)):

NOTE: *"If the correct inventor or inventors are not named on filing, a provisional application without a cover sheet under Section 1.51(c)(1), the later submission of a cover sheet under Section 1.51(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship." 37 C.F.R. Section 1.48(f)(2).*

NOTE: *"The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in Section 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(a)[,] application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.*

The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that Section 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C. 111(a) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111(a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

See 37 C.F.R. Section 1.53.

	GIVEN NAME	MIDDLE INITIAL OR NAME	FAMILY (OR LAST) NAME
1.	Jeffrey	Keller	TEUMER
2.	Jizeng		QIAO
3.	Erica		LaFONT
4.	Richard	Gregory	WOLOWACZ

2. Residence address(es) of the inventor(s), as numbered above (37 C.F.R. Section 1.51(c)(1)(iii)):

1. 213 Aspinwall Avenue, Brookline, Massachusetts 02446, United States
2. 47 Reed Street, Lexington, Massachusetts 02421, United States
3. 2 Daybrook Drive #125, Ayer, Massachusetts 01432, United States
4. Cowbrook Farm, Hurstbrook Close, Glossop, High Peak, Derbyshire SK13 8PL, United Kingdom

3. The title of the invention is (37 C.F.R. Section 1.51(c)(1)(iv)):
HAIR INDUCTIVE CELL CULTIVATION

4. The name, registration, customer and telephone numbers of the practitioner *(if applicable)* are (37 C.F.R. Section 1.51(c)(1)(v)):

Name of practitioner: Edward L. Adamson

Reg. No. 50,927 Tel. (617) 439-4444

Customer No. 21874

(complete the following, if applicable)

[ ] A power of attorney accompanies this cover sheet.

5. The docket number used to identify this application is (37 C.F.R. Section 1.51(c)(1)(vi)):

Docket No. 58392-P (70305)

6. The correspondence address for this application is (37 C.F.R. Section 1.51(c)(1)(vii)):
Linda M. Buckley, Esq.
Edwards & Angell, LLP, P.O. Box 9169, Boston, Massachusetts 02209

7. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. Section 1.51(c)(1)(viii)).

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

[X] No
[ ] Yes

The name of the U.S. Government agency and the Government contract number are:

_____

_____

8. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. Section 1.51(c)(2)-(3):

Specification: 13 pages
Drawings: 5 sheets

B. Additional documents:

[X] Claims: 3 pages No. of claims: 23

Note: See 37 C.F.R. Section 1.51.

[ ] Power of attorney
[ ] Small entity statement
[ ] Assignment
[X] Other: Return Postcard.

NOTE: Provisional applications may be filed in a language other than English as set forth in existing Section 1.52(d). However, an English language translation is necessary for security screening purposes. Therefore, the PTO will require the English language translation and payment of the fee mandated in Section 1.52(d) in the provisional application. Failure to timely submit the translation in response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 111(a) application is filed without providing the English language translation in the provisional application, the English language translation will be required to be supplied in every 35 U.S.C. 111(a) application claiming priority of the non-English language provisional application. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

9. Fee

The filing fee for this provisional application, as set in 37 C.F.R. Section 1.16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

[X] Applicant is a small entity.
[ ] Applicant is not a small entity.

NOTE: "A . . . statement in compliance with existing Section 1.27 is required to be filed in each provisional application in which it is desired to pay reduced fees." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,197.

10. Small entity statement

[ ] The statement(s) that this is a filing by a small entity under 37 C.F.R. Sections 1.9 and 1.27 is(are) attached.

11. Fee payment

[X] Fee payment in the amount of $80.00 is being made at this time.

[ ] No filing fee is to be paid at this time. (This and the surcharge required by 37 C.F.R. Section 1.16(l) can be paid subsequently.)

12. Method of fee payment

[X] Check in the amount of $80.00.
[ ] Charge Account No. _____, in the amount of $ _____.
A duplicate of this Cover Sheet is attached.

Please charge Account No. 04-1105 for any fee deficiency.

_____
Signature of submitter

OR

Date: November 14, 2002

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Customer No.: 21874

PATENT TRADEMARK OFFICE

BOS2_318812.1

Signature of practitioner

Edward I. Adamson
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Hair Inductive Cell Cultivation

The present invention relates to culturing cells for use in hair induction. In particular, the invention relates to a method for cultivating hair inductive cells (for example dermal papilla and/or dermal sheath cells), cells cultivated according to method, and use of these cultivated cells.

Hair loss affects millions of people, including over 40% of men over the age of 30 and a significant number of women. Many people seek remedy in a variety of pharmaceuticals and other treatments including for example locally-delivered Minoxidil™ (Pharmacia) and orally-delivered Propecia™(Merck). One solution to hair loss is hair follicle transplantation, a procedure in which hairs from a non-balding region of the scalp are transplanted to bald areas. The follicles from the non-balding region retain their low susceptibility to androgens even in their new location. However, this procedure is limited by the relatively small number of hair follicles that can be harvested from the non-bald region and "donated" to the hairless region. A complete pattern of hair with equivalent density of follicles to that typical for example of teenagers cannot be readily obtained using hair follicle transplantation.

Dermal papilla cells (DP cells) can be removed from hair follicles (e.g. derived from non-balding sites) and directly transplanted to another place in the skin where they will instruct the skin to form new hair follicles (Oliver, R. F. (1967) J. Embryol. Exp. Morphol. 18(1): 43-51). This procedure could be developed into an alternative therapy for hair restoration. However, an impediment to the development of this alternative therapy has been that it is subject to the same limitation on the availability of donor follicles.

DP cells can expand in number when placed into culture using conventional conditions but under these conventional conditions they rapidly lose their ability to induce new hair formation (Jahoda, C. and R. F. Oliver (1981) Br. J. Dermatol. 105(6): 623-7; Messenger, A. G. (1984) Br. J. Dermatol. 110(6): 685-9). Something present *in vivo* that preserves DP cell hair inductive ability is therefore lacking in conventional cell culture

medium. A hair transplantation process using DP cell transplantation is not commercially viable without a culture method which allows expansion of cell number without loss of inductive ability.

Recently, two methods have been described that are able to support expansion of cell number while also maintaining the hair inductive potential of DP cells. In a first method, conditioned medium (CM) collected from mammalian epidermal cells (keratinocytes), or co-culture with keratinocytes, were shown to support both expansion of DP cell number and maintenance of the hair inductive phenotype over several culture passages. This first method is described in USP 5,851,831 and in J. Invest. Dermatol. 111: 767-75 (1998).

A second method describes the culture of dermal papilla cells in the presence of an increased level of *wnt* protein or an agent that mimics the effects of *wnt*-promoted signal transduction (see International Patent Application No. PCT/US01/10164 published as WO01/74164 and Kishimoto et al., Genes & Development 14: 1181-85 (2000)). In this second method, a *wnt* protein or a functional fragment or analogue thereof is added to the culture medium as a purified product, or by expressing a recombinant protein in producer cells and providing the *wnt* protein in medium conditioned by the growth of *wnt* producer cells, or by co-culture with producer cells.

Some problems with the known methods are of a practical nature relating to regulatory issues, manufacturing costs, and technical problems. For the first method, normally a manufacturer will cryopreserve a large number of cells to provide a bank of cells with identical properties that can be used in manufacture. Cells from this bank will need to undergo stringent tests for safety, including tests that screen for infectious agents that could potentially cause disease in human recipients. Eventually, the cell bank will be depleted and will need to be replenished, and the new bank will require new testing. In the case of a cell strain derived from primary tissue, such as sole skin keratinocytes that have a limited life span in culture, the size of the cell bank will be limited by the ability of the cells to grow in culture, so the costs associated with the use of such a tissue source will be greater than they would be if the original cell were available in unlimited supply. In addition, there can be variations between the donors of the original cell strains that

may result in irreproducible manufacturing conditions. Use of an alternative cell source, such as those described herein, provides an unlimited source of cells with uniform properties that only have to be tested once.

In the second method described above, where CM from a recombinant *wnt* producing cell is used to promote DP cell growth, similar testing would be minimally required. However, because the cells are producing recombinant protein, additional safety tests are required. The stability of *wnt* gene expression in these producer cells is also a potential problem, and it may be necessary to re-derive or sub-clone the cells in the event *wnt* gene expression is lost or reduced over time in culture.

The present invention address problems associated with the prior art by allowing growth of an alternative cell source with uniform properties.

According to a first aspect of the present invention there is provided a method for cultivation of hair inductive cells, comprising the step of culturing the hair inductive cells in a culture medium comprising, or consisting essentially of, a medium conditioned by cells derived from non-epidermal tissue.

In contrast to the culture medium described in USP5,851,831 and WO01/74164, the culture medium of the present invention may be prepared from a stable cell line that is available in a virtually unlimited supply. Alternatively, the culture medium may be prepared from a non-epidermal tissue-derived cell strain that is more readily available or more convenient to culture. The method allows for long-term culture of hair inductive cells while keeping the original capability (i.e. hair inductive potential) of the cells intact.

The ability of a medium conditioned by cells derived from non-epidermal tissue to maintain hair inductive phenotype of hair inductive cells was highly unexpected. In contrast to USP5,851,831, where keratinocytes that would interact *in vivo* with epidermal cells to form hair cells, the non-epidermal tissue-derived cells used in the present invention are not necessarily associated *in vivo* with hair inductive cells. Thus the present invention shows that various terminally differentiated cells or committed progenitor cells which are not epidermal may be used in place of keratinocytes to

condition medium to retain the hair inductive potential of hair inductive cells during the culture expansion phase important in any process to produce large number of hairs, for example from a small biopsy containing relatively few follicles.

The non-epidermal tissue may be of ectodermal (for example epithelial and/or neuronal), and/or endodermal, and/or mesodermal origin.

In one embodiment of the process for use of the invention proposed herein, medium conditioned by the growth of certain lineages of cell types of ectodermal but not epidermal origin. It is surprising that cells of epithelial origin from "non-hairy" epithelia which are not normally found in close proximity to hair inductive cells such as DP cells, unlike the keratinocytes used in USP 5,851,831 can nevertheless provide similar cues to the hair inductive cells.

It is also surprising that conditioned medium from cells of neural origin would provide the correct cues to DP cells for them to retain their inductive potential.

In another embodiment the invention uses conditioned medium derived from cells of endodermal origin. The ability to use endodermal-derived cells is surprising as it represents a cell type from a different embryological layer.

The hair inductive cells may be dermal papilla (DP) cells and/or dermal sheath (DS) cells.

The conditioned medium may be obtained using a cell line. Suitable cell lines may be more readily available or more convenient to culture compared with existing methods for proliferating hair inductive cells. For example, the cell line may be either one of the breast epithelial cell lines MDA-MD-230 or MCF-7.

The cell line may be derived from a donor that has been screened and tested for risk factors associated with transplantation.

The culture medium may be free of recombinant genes and/or recombinant products thereof. The culture medium may be free of viral vectors. Recombinant genes and their products and viral vectors may raise safety concerns that are avoidable using the present

method.

The conditioned medium may be frozen prior to use.

When sub-culturing hair inductive cells, the culture medium may be completely changed or the cells may be split fed by changing only part of the medium for example by adding fresh conditioned medium.

The conditioned medium may generated from cells grown in serum-free medium. The potential problem of transmission of infectious agents potentially present in serum has led to the use of closed herds for generating serum used in the manufacture of cellular products. These infectious agents include bovine spongiform encephalopathies (BSE). There may be significant regulatory advantages for a process a totally free of the use of serum.

In one embodiment, the non-epidermal cells are cultured in an appropriate defined medium. Defined media will be known to those skilled in the art, suitable for the propagation and culture of each cell type which may be used for the generation of conditioned medium described in this invention.

The conditioned medium may have a serum-free component with a total protein content above 10 μg/ml, for example above 100 μg/ml or above 1 mg/ml. In serum-containing medium, serum will normally be the major protein component.

The conditioned medium may concentrated (for example, by ultrafiltration) prior to use. This will allow for concentration of factors necessary to maintain hair inductive phenotype.

The method may further comprise the step of sub-culturing the hair inductive cells in the culture medium for three or more passages, for example seven or more passages. For example, the hair inductive cells undergo about 30 population doublings before use.

The method may be suitable for long-term culture of hair inductive cells. Long term culture may be defined by either passage number or more properly by population

doublings. Primary fibroblasts, keratinocytes, and satellite cells can sometimes be expanded for up to 120 population doublings from young donors. For example, when fibroblasts are split 1 in 3 during passage they can reach up to passage 40 before becoming senescent. However, if the plating density used was lower then they would undergo more population doublings between each passage. In absolute terms this would be a long-term culture. Sufficient hair inductive cells (for example, DP cells) for transplantation of a full pattern of hair may be obtained from less than 30 follicles by passage up to p3 (passage 3) using optimal plating density for the cells.

The method may further comprise the step of harvesting or isolating cultured or subcultured hair inductive cells.

The hair inductive cells may be allogeneic to the non-epidermal tissue. Alternatively, the hair inductive cells may be autologous to the non-epidermal tissue.

In a further aspect of the invention, there is provided a method of long term cultivation of dermal papilla (DP) cells and/or dermal sheath (DS) cells of a mammalian species, the method comprising the steps of culturing and sub-culturing the DP and/or DS cells in a cell culture medium which consists essentially of, or is supplemented with, a medium conditioned by one or more mammalian cells derived from a non-epidermal tissue, thereby proliferating the DP and/or DS cells while preserving their hair inductive potential.

In another aspect of the invention there is provided a method of providing and maintaining dermal papilla (DP) and/or dermal sheath (DS) cells for transplantation, the method comprising the steps of obtaining a DP and/or DS cell from a subject and culturing the DP and/or DS cell under conditions described herein.

In any of the methods described herein, the culture medium may further comprise the cells derived from non-epidermal tissue. In this embodiment, the conditioned medium may be produced "*in situ*" by the non-epidermal tissue as the tissue grows in the presence of the hair inductive cells.

According to another aspect of the present invention there is provided a method for cultivation of hair inductive cells, comprising the step of culturing the hair inductive cells in a co-culture system, whereby the hair inductive cells are provided necessary factors for the maintenance of hair inductive phenotype by feeder cells. These feeder cells are preferably in the same culture vessel. In such a co-culture system, the feeder cells, which are cells that provide the factors to the hair inductive cells, provide culture support either through intermingling with the hair inductive cells or through a membranous barrier where the cells are kept separate but share the same culture medium. The feeder cells are preferably derived from non-epidermal tissue.

Where the two cell types are cultured in an intermingled arrangement, the feeder cells may be mitotically inactivated so they cannot divide and will therefore not significantly contaminate the hair inductive cells for transplantation. Mitotic inactivation may be accomplished by drug treatment or by irradiation, techniques known to those skilled in the art. Where a membranous barrier is used, the feeder cells may be separated from the hair inductive cells by a permeable membrane that allows the exchange of medium but does not allow cell types to contact each other. This prevents contamination of hair inductive cells by the feeder cells. Examples of co-culture system that use a membrane barrier are transwell plates and Boyden chambers and are known to those skilled in the art.

In another aspect of the invention there is provided cultured hair inductive cells, DP cells or DS cells obtainable using any of the methods described herein.

Also provided is the use of these cultured hair inductive cells, DP cells or DS cells for the treatment of male pattern baldness.

Further provided is the use of these cultured hair inductive cells, DP cells or DS cells in the production of *in vitro* skin equivalents.

In a further aspect of the invention there is provided a composition comprising hair inductive cells and a culture medium comprising a medium conditioned by cells derived from non-epidermal tissue.

Conditioned medium, i.e. the medium conditioned by cells derived from non-epidermal tissue, may be defined as a spent medium obtained by the growth of living cells in culture medium. Conditioned medium contains numerous secreted factors expressed and secreted by the cultured living cells into the culture medium. These secreted factors would include numerous molecules and macromolecules (proteins, glycoproteins such as growth factors, proteases, soluble receptors, hormones, etc.) as well as waste products produced by the cultured cells. Different lineages of cells express different phenotypes and will therefore secrete different sets of factors, and/or factors at different concentrations, into the culture medium. Therefore, a conditioned medium from a fibroblast culture for example will contain different molecules in different concentrations to a conditioned medium from a keratinocyte culture.

Typically living cells may be grown in a monolayer culture in a variety of containers including on the surface of standard tissue culture flasks (e.g. T175 or T75), roller bottles or on micro-carrier beads. Conditioned medium may include one or more known basal media e.g. DMEM, Chang's, Hams F12, etc. Conditioned medium may also be derived from three dimensional cell cultures or culture of a tissue or organ. Conditioned medium may be derived from culture in both serum free or serum supplemented media. Optimisation of the length of time of culture require for cells to secrete factors at sufficient concentration and/or the volume of culture medium to be used for a given number of cells may be developed empirically by those skilled in the art. In general terms, the higher the number of cells for a given volume, and the longer the cells are cultured to produce a conditioned medium, the higher the concentration of "secreted factors" typical of the cell lineage.

Conditioned medium may be tested for activity according to the invention as described in the Experimental section below. It may be desirable to adopt a quality assurance approach in closely defining all procedures to ensure manufacture of a conditioned medium is consistent. For example, cell plating density, feeding regimes, volume of culture medium, basal medium and/or supplements could be accurately defined and applied.

In one embodiment of the invention, the hair inductive cell culture obtained, i.e. the "product" derived, is autologous. DP or DS cells are taken from one biopsy. Specific non-epidermal cells are derived from another biopsy and cultured so as to provide the conditioned medium. This conditioned medium is added to the DP or DS cells to allow them to retain the inductive potential. Typically the DP or DS cells can be derived from a skin biopsy of 1-2 cm^2 containing on average 400 follicles and is expanded (for example, to passage 3) to obtain sufficient cells for DP cell transplantation by the hair surgeon or otherwise.

In another embodiment of the invention, only the DP and/or DS cellular component is autologous. The DP and/or DS cells are obtained as above from a biopsy. However the non-epidermal cells from which the conditioned medium is derived are obtained from a separate source (preferably an allogeneic or xenogeneic source). This source would preferably be from a validated cell bank which has been screened for adventitious agents. Because these cells are stored as master cell banks it may be possible to manufacture large amounts of conditioned medium using known tissue culture methods. In this case, the conditioned medium could then be concentrated via standard protein methods e.g. ultrafiltration and stored as a concentrated stock, to be added to the DP and/or DS cells as a supplement as appropriate. This type of approach has certain advantages in that if a large batch of conditioned medium is prepared, a small sample can be tested for its ability to allow DP and/or DS cells to retain their inductive potential in a hair induction assay. Because hair induction assays are time consuming, there may be further advantages in confidence in use of the expanded DP and/or DS cells to using an additive from batch with a quality control (QC)-tested activity. This contrasts with the method of USP 5,851,831 in which keratinocyte conditioned medium may be used without knowing from a QC test whether the activity causing the retention of hair inductive capability of DP and/or DS cells has been retained.

In a further embodiment of the invention, the DP and/or DS cells to be expanded may be derived from an allogeneic source. This may be used in conjunction with conditioned medium derived from allogeneic source as described above. The DP and/or DS cells would by preference also be from a cell source screened for the presence of adventitious

agents and from a source approved by appropriate regulatory agencies.

The invention therefore addresses the previous block to the development of a reproducible, cost-effective process for commercialisation of hair inductive cell (for example DP and/or DS cell) transplantation

Specific embodiments of the invention will be described below with reference to the accompanying figures, of which:

Fig. 1 is a photograph showing dermal papilla cells soon after isolation;

Fig. 2 is a photograph showing dermal papilla cells grown in keratinocyte CM;

Fig. 3 is a photograph showing dermal papilla cells grown in CM collected from human dermal fibroblast (HDF) cells;

Fig. 4 is a photograph showing dermal papilla cells grown in CM prepared from a first breast epithelial cell line, MDA-MB-230; and

Fig. 5 is a photograph showing dermal papilla cells grown in CM prepared from a second breast epithelial cell line, MCF-7.

Experimental

The experiments described below demonstrate cultivation of DP cells in different culture media to show that growth in culture medium (CM) of the present invention results in maintenance of morphology associated with hair inductive capability.

Materials and Methods

Preparation of Conditioned Medium

When cells grown for test CM reached a near-confluent stage, fresh medium was applied. After 3 days, the medium was removed and filtered through a 0.22 μm filter to sterilize it and to remove cells and debris. The conditioned medium was combined with Chang medium at a 1:1 (volume: volume) ratio and used to feed cultured dermal papilla cells.

Cell Lines

The following cell types of ectodermal origin were tested: 2 human breast tumor cell lines; MCF-7 (American Type Culture Collection (ATCC) and MDA-MB-230 (ATCC); human neural cells, and human epidermal keratinocytes (HK). The following cell types of mesodermal origin were tested: human dermal fibroblasts, skeletal muscle cells, prostate epithelial cells, renal epithelial cells, endodermal cells, and immune system cells. The following cell types of endodermal origin were tested: hepatocytes, airway epithelium, and bladder epithelium. All cell types were cultured under conditions recommended by the suppliers. HDF and HK were isolated from freshly excised human tissue and grown under standard conditions.

Human DP Cell Culture

Human DP were placed into culture after microdissection of DP from hair follicles. After one passage in standard medium, cultures were replated to provide enough culture dishes to test each conditioned medium. Cultures were fed every 2-3 days with fresh conditioned medium:Chang medium and passaged when confluent. Cultures were maintained in this

manner for several passages. All cells shown in Figures 2-5 were photographed at passage 7.

Assay for Hair Induction

The chamber assay (Weinberg et al., J Invest. Dermatol 100: 229-236 (1993)) is used to test for hair induction by the cultured hair inductive (for example, DP) cells. Circular, full-thickness wounds are made on the backs of athymic mice and a graft chamber was placed into the wounds. Rodent keratinocytes mixed with cultured dermal papilla cells or dermal fibroblasts as a control are injected into the graft chambers. After one week the chambers were removed and the wounds were bandaged. The engrafted cells form full-thickness skin and, if hair-inductive dermal papilla cells are present, new hair follicles also form. The new hairs induced by the DP cells appear within 4 weeks of grafting.

A variation of the chamber assay allows a quantitative assessment of hair inductive potential. DP cells can be induced to aggregate under certain circumstances. Cell aggregates can be prepared and mixed with a single cell suspension mixture of rat keratinocytes and human fibroblasts and introduced into the chamber assay. In this manner, the pre-aggregated DP are the only hair-inductive dermal cells in the graft. If a predetermined number of DP cell aggregates is introduced into the chamber, a quantitative assessment of hair inductive is made by counting the number of hair follicles in the graft after a period of 3 to 4 weeks. In this manner, the hair inductive potential of DP cells cultured under different conditions is quantitatively compared. This assay is used to compare DP cells grown using the various CM described above.

Results and Discussion

Dermal papilla cells have a characteristic morphology when first isolated. The cells are small and round or polygonal and grow in loose clusters (Figure 1). Within some early cultures, there are also large, flat cells that do not divide. Over time in culture, the proportion of large, flat cells increases. These cells have a reduced ability to induce hair follicle formation. This characteristic morphology observed under ideal culture conditions correlates with hair induction. Loss of this characteristic morphology and an

increase in the proportion of large cells correlate with the inability to induce hair follicles.

When DP cells are cultured using keratinocyte-derived CM (as described in USP 5,851,831) the characteristic morphology is maintained over numerous passages. Figure 2 shows a culture of DP cells grown to passage 7 in keratinocyte CM. In this medium, the cells maintain their morphology as well as their ability to induce hair formation.

DP grown in CM collected from HDF convert to a more fusiform morphology, looking much more like normal fibroblasts (Figure 3). This morphology is not consistent with hair induction.

As shown in Figure 4, cells grown in CM from MDA-MB-230, a breast epithelial cell line, are close in morphology to the keratinocyte CM grown cells, as are the cells grown in CM from another breast epithelial cell line, MCF-7, shown in Figure 5.

The interaction between keratinocytes, an epithelial cell type, and DP cells, a mesenchymal cell type, is similar to other epithelial-mesenchymal interactions in the body. Epithelial-mesenchymal interactions are fundamental processes that occur during embryogenesis and they contribute to the formation of many organs and other structures in the body. These interactions are mediated by signaling molecules produced by one cell type that instruct the other cell type to respond in a characteristic manner. The present invention demonstrates that, surprisingly, keratinocytes can be replaced by another cell type that produces the same or similar signaling molecule in order to obtain the response in a mesenchymal cell. These other cell types as described herein are thus able to maintain DP cells in a hair inductive state in culture.

Claims

1. A method for cultivation of hair inductive cells, comprising the step of culturing the hair inductive cells in a culture medium comprising a medium conditioned by cells derived from non-epidermal tissue.

2. The method of claim 1, wherein the culture medium consists essentially of the conditioned medium.

3. The method of either claim 1 or claim 2, in which the non-epidermal tissue is of ectodermal (for example, epithelial and/or neuronal) and/or endodermal and/or mesodermal origin.

4. The method of any preceding claim, in which the hair inductive cells are dermal papilla (DP) cells and/or dermal sheath (DS) cells.

5. The method according any preceding claim, in which the conditioned medium is obtained using a cell line.

6. The method according to claim 5, in which the cell line is either one of the breast epithelial cell lines MDA-MD-230 or MCF-7.

7. The method according to claim 5, in which the cell line is derived from a donor that has been screened and tested for risk factors associated with transplantation.

8. The method of any preceding claim, in which the culture medium is free of recombinant genes and/or recombinant products thereof.

9. The method of any preceding claim, in which the culture medium is free of viral vectors.

10. The method of any preceding claim, in which the conditioned medium is frozen prior to use.

11. The method of any preceding claim, in which the conditioned medium has a serum-free component with a total protein content above 10 μg/ml, for example above 100 μg/ml or above 1 mg/ml.

12. The method of any preceding claim, in which the conditioned medium is concentrated (for example, by ultrafiltration) prior to use.

13. The method of any preceding claim, further comprising the step of subculturing the hair inductive cells in the culture medium for three or more passages, for example seven or more passages.

14. The method of any preceding claim, further comprising the step of harvesting or isolating cultured or subcultured hair inductive cells.

15. The method of any preceding claim, in which the hair inductive cells are allogeneic to the non-epidermal tissue.

16. The method of any of claims 1 to 5 or 7 to 14, in which the hair inductive cells are autologous to the non-epidermal tissue.

17. A method of long term cultivation of dermal papilla (DP) cells and/or dermal sheath (DS) cells of a mammalian species, the method comprising the steps of culturing and sub-culturing the DP and/or DS cells in a cell culture medium which consists essentially of, or is supplemented with, a medium conditioned by one or more mammalian cells derived from a non-epidermal tissue, thereby proliferating the DP and/or DS cells while preserving their hair inductive potential.

18. A method of providing and maintaining dermal papilla (DP) and/or dermal sheath (DS) cells for transplantation, the method comprising the steps of obtaining a DP and/or DS cell from a subject and culturing the DP and/or DS cell under conditions described in any of claim 1 to 17.

19. The method of any preceding claim, in which the culture medium further comprises the cells derived from non-epidermal tissue.

20. Cultured hair inductive cells, DP cells or DS cells obtainable using the method of any of claims 1 to 19.

21. Use of the cultured hair inductive cells, DP cells or DS cells of claim 20 for the treatment of male pattern baldness.

22. Use of the cultured hair inductive cells, DP cells or DS cells of claim 20 in the production of *in vitro* skin equivalents.

23. A composition comprising hair inductive cells and a culture medium comprising a medium conditioned by cells derived from non-epidermal tissue.

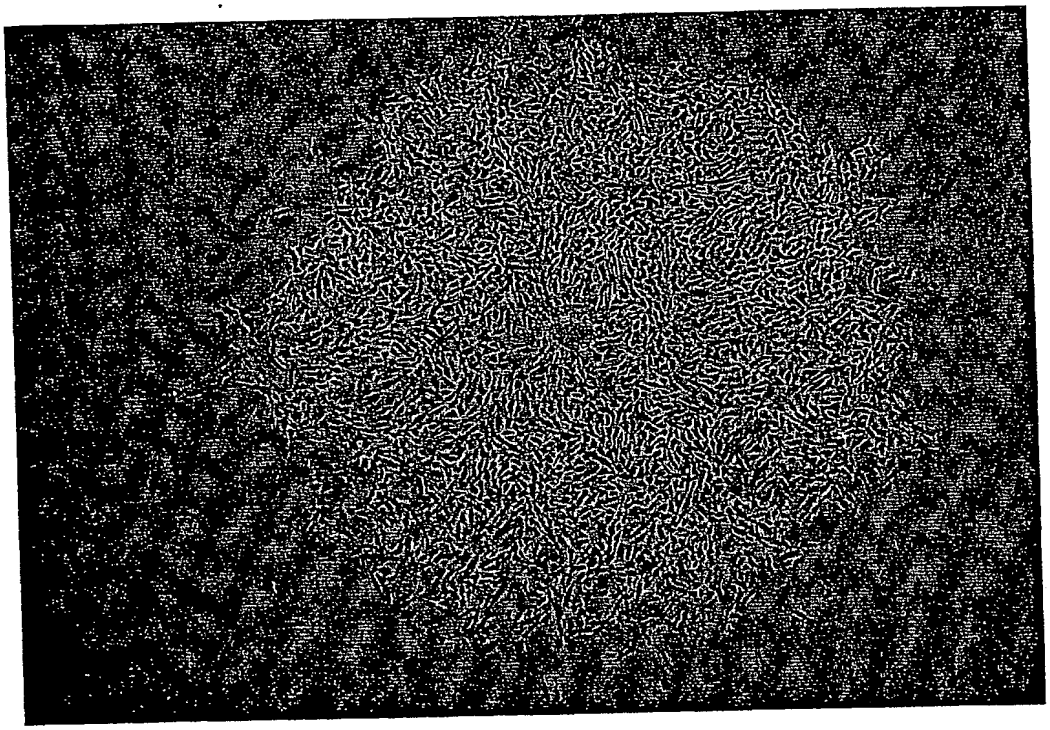

Fig. 1

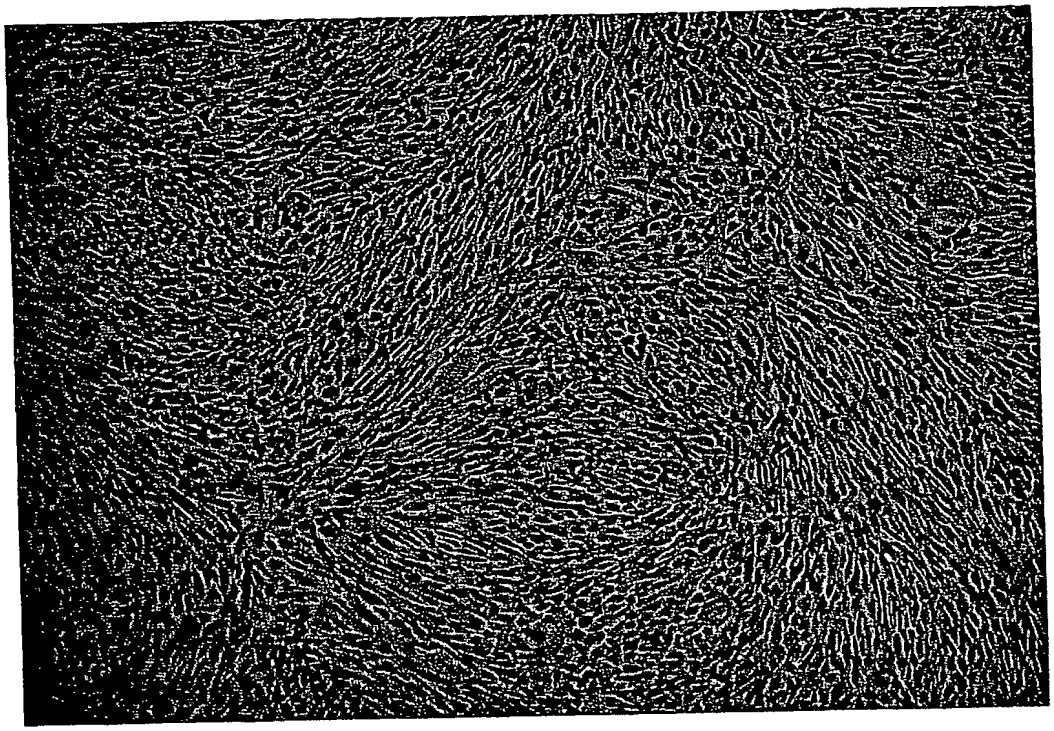

Fig. 2

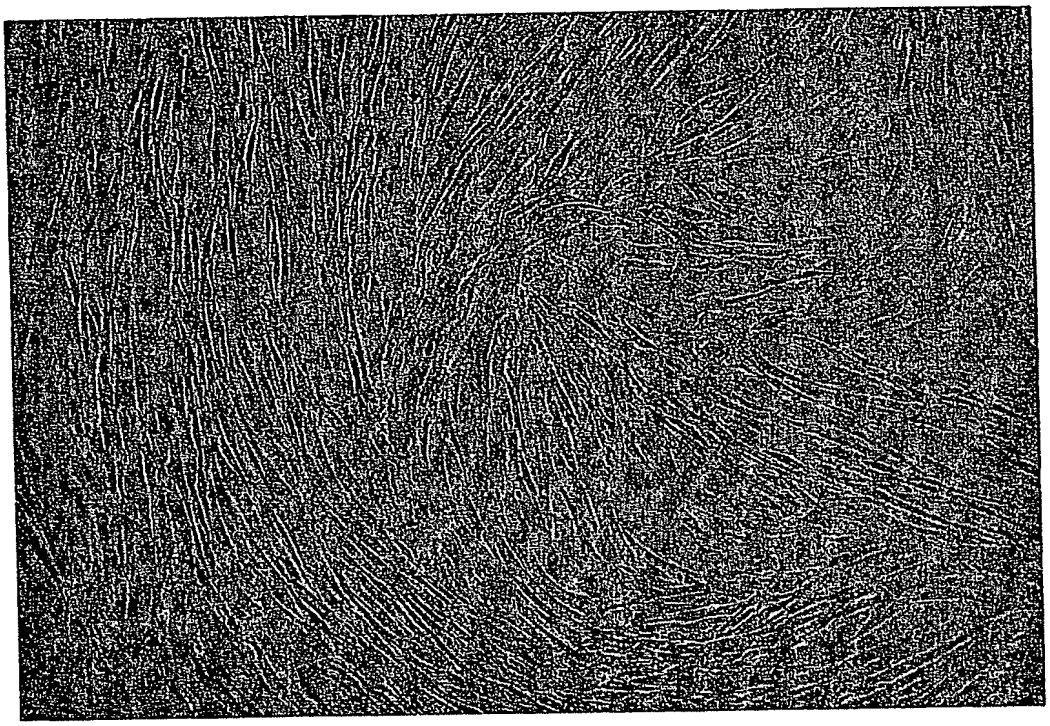

Fig. 3

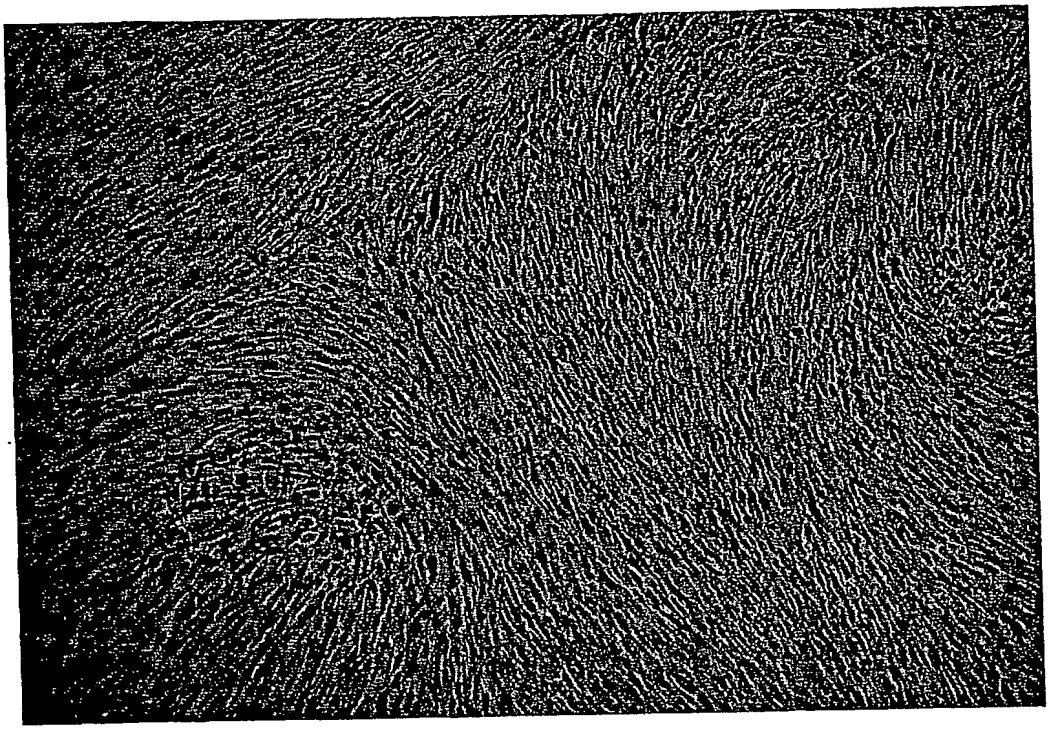

Fig. 4

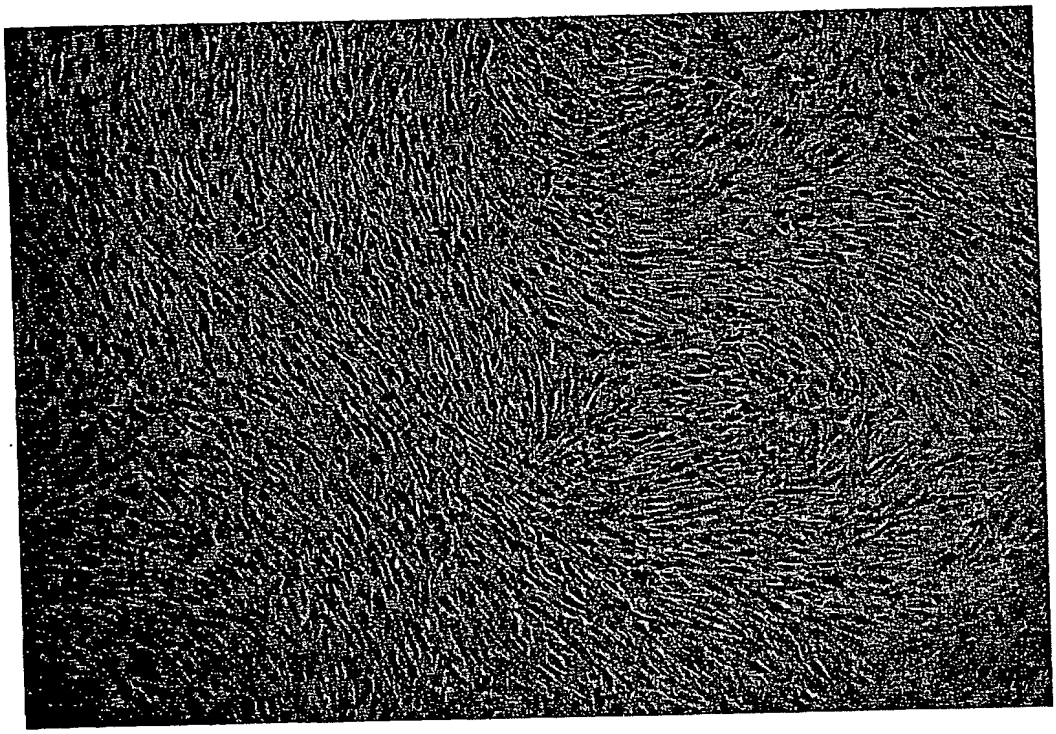

Fig. 5